Plasma MMP-9 – a Marker of Carotid Plaque Instability†

I. M. Loftus*, A. R. Naylor, P. R. F. Bell and M. M. Thompson

Department of Surgery, Leicester University, Leicester, U.K.

Objectives: to investigate whether peripheral blood levels of matrix metalloproteinases (MMPs) or their inhibitors are altered in patients with particulate cerebral embolisation.

Design: a prospective study.

Materials and methods: using sandwich enzyme immunoassay, plasma levels of MMPs-1, -2, -3 and -9, plus TIMPs-1 and -2 were quantified in 70 consecutive patients undergoing carotid endarterectomy. Patients were monitored with transcranial Doppler (TCD) preoperatively and during the dissection phase of the operation to detect those with spontaneous particulate embolisation (n = 21).

Results: the plasma level of MMP-9 was significantly higher in those patients with evidence of spontaneous embolisation compared to those without. There were no differences in other MMP levels, or plasma concentrations of TIMPs.

Conclusions: plasma MMP-9 levels are elevated in patients with particulate cerebral embolisation, and this may represent a novel marker of atherosclerotic plaque instability.

Key Words: MMP-9; Gelatinases; Plaque instability; Embolisation; Transcranial Doppler.

Introduction

The atherosclerotic plaque is a dynamic structure, undergoing continuous remodelling of the extracellular matrix upon which its structural integrity depends. Acute changes within the plaque are a prelude to the onset of clinical ischaemic events. If the plaque undergoes acute disruption such as rupture or ulceration, exposure of highly thrombogenic material in the plaque core leads to thrombus formation. Secondary thromboembolism may then cause stroke. There is considerable evidence to support the theory that acute plaque changes immediately precede the onset of clinical symptoms. Histological studies have shown that plaque rupture, intra-plaque haemorrhage and overlying thrombus are significantly more common in patients with recent symptoms compared to those without. Significant disease progression is three times more likely to be identified on Duplex ultrasound in patients immediately after an ischaemic event than in patients who have remained asymptomatic.

Recent work suggests that each phase of the atherosclerotic process may be mediated by a series of enzymes called matrix metalloproteinases or MMPs.14 The main physiological regulators of the extracellular matrix. Secreted in a latent pro-enzyme form by a range of cell types, including inflammatory cells, fibroblasts and smooth muscle cells, they require activation by limited proteolysis. There is evidence linking MMPs to disease states in which tissue degradation plays a key role, including aneurysmal disease, cancer, and arthritis.12 Their role in pathological states has led to the development of specific inhibitors, some of which are currently involved in clinical trials.13

Initial plaque disruption has been shown to occur predominantly in the shoulder of the plaque, an area rich in macrophages.14 The production of MMPs in this region has been shown to be significantly higher than in other parts of the plaque.15 MMP activity in coronary plaques has been shown to correlate closely with the onset of unstable angina, and of particular interest are the gelatinases, MMP-2 and MMP-9.

MMP-2 is constitutively expressed in vascular smooth muscle cells in normal arteries and plaques exhibit increased levels of expression. Plaques prone to acute disruption also exhibit induction and activation of MMP-9 in smooth muscle cells and inflammatory macrophages. More recently, the level of MMP-9 has been shown to be four times higher in the most unstable plaques based on symptomatology, embolisation and histological features of plaque instability.16 There are no systemic markers or investigations that can successfully predict those plaques...
most at risk of disruption towards which pharma-
cotherapy could be targeted.

Reports have suggested that the levels of MMPs
are elevated not only in affected tissue but also in
peripheral blood in patients with cancer, arthritis and
more recently acute coronary syndromes.17,18 This
raises the possibility that patients at risk of acute
plaque disruption may have elevated blood levels of
MMPs.

The aim of this study was to investigate the plasma
levels of the major MMP and TIMP (tissue inhibitor
of MMP) subtypes in patients undergoing carotid en-
derterectomy to ascertain any differences in those with
spontaneous particulate embolisation, probably the
most sensitive indicator of plaque instability.19

Materials and Methods

Seventy-five consecutive patients admitted for carotid
endarterectomy (CEA) into a single vascular surgical
unit were entered into this study. Local ethical com-
mittee approval was obtained for the procurement of
specimens and all patients gave full informed consent
for the study. A clinical history was obtained from
each patient, with particular care taken to establish
the number and duration of ischaemic events. All
patients underwent a thorough neurological ex-
amination. Focal cerebral ischaemic events were
defined as transient ischaemic attack, amaurosis fugax,
central retinal artery occlusion or cerebrovascular ac-
cident. All patients were on long-term aspirin therapy
(75 or 150 mg daily) and underwent a pre-operative
Duplex ultrasound assessment of the carotid plaque
for quantification of the degree of stenosis.

Preoperatively patients were monitored with trans-
cranial Doppler (TCD) for 30 min, plus intraoperatively
during the dissection phase of the operation. This
aimed to identify those with ongoing particulate micro-
embolisation, highly indicative of plaque instability.19
Continuous TCD monitoring of the ipsilateral middle
cerebral artery was performed using a SciMed PC Dop
842 TCD (SciMed, Bristol, U.K.). Signals were recorded
onto digital audio-tape for off line analysis and in-
terpretation of embolic signals as described pre-
viously.20 For the purpose of this study, emboli were
only recorded if they occurred in the preoperative
period or the dissection phase of the operation and,
after internal carotid artery clamping, recording was
discontinued. This was to ensure that all recorded
emboli represented spontaneous dislodgement of par-
cticular matter from the plaque rather than air emboli
and/or thrombus relating to the surgical procedure.

Blood sampling and immunoassay

Blood samples were drawn from the peripheral vein
of each patient 24 h preoperatively. For the preparation
of plasma 2Na-EDTA (final 0.1%) was added to whole
blood. Following centrifugation, plasma was siphoned
into freezing vials, snap frozen in liquid nitrogen and
stored at −80°C. MMP and TIMP quantification was performed using
ELISA techniques for MMP-1, MMP-2, MMP-3, MMP-
9, TIMP-1, TIMP-2 and TIMP-1/MMP-1 complex using
Biotrak assay systems (Amersham, Amersham, Bucks,
U.K.), validated for use with human plasma.17 These
provide a specific and precise quantitative de-
termination of enzyme levels and are based on a two-
site ELISA sandwich format, employing two antibodies
directed against different epitopes of the enzyme.

During the first incubation step, MMP present in
the tissue homogenate binds to a microtitre plate
precoated with antibody. During the second incubation
step, detection antibody coated with horseradish per-
oxidase (HP) is added, which forms an immobilised
complex. The amount of peroxidase bound to each well
is determined by the addition of tetramethylbenzidine
(TMB). The reaction is stopped by the addition of an
acid solution and the resultant colour measured at
450 nm in a microtitre plate spectrophotometer. The
concentration of enzyme is determined by in-
terpolation from a standard curve.

The MMP-2 and MMP-9 assays recognise free and
TIMP complexed pro-enzyme only, showing no cross-
reactivity with activated forms of the enzyme nor other
MMPs. The MMP-1 and MMP-3 assays recognise total
enzyme both active and latent, plus enzyme complexed
with TIMPs. The TIMP-1/MMP-1 complex assay re-
ognises activated MMP-1 that has subsequently been
complexed with TIMP-1, but not free MMP-1 or TIMP-
1. The TIMP-1 assay recognises all TIMP-1, both free
and complexed with MMPs, while the TIMP-2 assay
detects all TIMP-2 except that bound to pro-MMP-2.

Statistical analysis

All results are expressed as median values and inter-
quartile ranges. Risk factors were analysed using the
chi-squared test, whilst differences in MMP levels
were analysed using the non-paired, non-parametric
Mann–Whitney U-test. Significance was assumed with
a p value <0.05. Logistic regression analysis was per-
formed using the SPSS 8.0 statistical package and the
individual risk factors expressed as a relative risk.
Table 1. Demographics of patient groups. There were no significant differences between those patients with emboli compared to those without.

<table>
<thead>
<tr>
<th></th>
<th>Emboli positive</th>
<th>Emboli negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n=21 )</td>
<td>( n=49 )</td>
</tr>
<tr>
<td>Age: median (range)</td>
<td>68 (46–80)</td>
<td>68 (46–82)</td>
</tr>
<tr>
<td>Male:Female ratio</td>
<td>7:14</td>
<td>18:31</td>
</tr>
<tr>
<td>Smoker – current</td>
<td>3 (14%)</td>
<td>17 (35%)</td>
</tr>
<tr>
<td></td>
<td>5 (24%)</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>IHD</td>
<td>14 (67%)</td>
<td>33 (67%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>14 (62%)</td>
<td>31 (63%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5 (24%)</td>
<td>9 (18%)</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>3 (14%)</td>
<td>11 (22%)</td>
</tr>
</tbody>
</table>

Results

Five patients were excluded from the study because they had no transcranial window, preventing the detection of emboli. Of the remaining 70 patients, spontaneous particulate embolisation was detected in 21, with 49 showing no evidence of embolisation. The median number of emboli detected in the emboli positive patients was 4 (range 1–16). The clinical characteristics of these two groups are shown in Table 1. There were no significant differences between the groups on the basis of known risk factors for atherosclerosis or regular medications.

All had >70% carotid stenosis on Duplex scanning with no difference between the groups in the percentage stenosis.

Figure 1 demonstrates the peripheral blood levels of MMP-9 in patients with or without evidence of spontaneous particulate embolisation. The level of MMP-9 detected on ELISA was significantly higher in patients with embolisation than those without (\( p = 0.03 \), Mann–Whitney \( U \)-test). In the non-embolising group, the median MMP-9 level was 26 ng/ml (iqr 19–40 ng/ml). This is very similar to the levels previously reported in normal volunteers. In the embolising group the level was much higher, with a median concentration of 44 ng/ml (iqr 31–59 ng/ml).

The plasma levels of MMP-2 in both groups were very similar (median concentration 921 ng/ml for no emboli vs. 991 ng/ml for those with emboli) and close to previously published levels for patients with coronary atherosclerosis.

The circulating levels of MMP-1, MMP-1/TIMP-1 complex and MMP-3 were very low, with no difference between those patients who were embolising and those not embolising (Table 2).

Whilst quite high concentrations of both TIMP-1 and TIMP-2 were detected in plasma of embolising and non-embolising patients, there were no significant differences between the groups in either inhibitor (Table 2).

Logistic regression analysis was performed to assess whether the plasma level of MMP-9 represented an independent predictor of embolisation. Taking all other atherosclerotic risk factors into account and the presence of neurological symptoms, plasma MMP-9 levels represented the only significant predictor of embolisation (\( p = 0.05 \)). A plasma MMP-9 level of greater than 30.2 ng/ml (the median value for the entire patient group) equated to a relative risk of 2.54 (Table 3). Whilst the presence of

---

Table 2. The concentrations (ng/ml, median (iqr)) of the major MMP and TIMP subtypes excluding MMP-9. There were no significant differences between the groups.

<table>
<thead>
<tr>
<th></th>
<th>Emboli positive</th>
<th>Emboli negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n=21 )</td>
<td>( n=49 )</td>
</tr>
<tr>
<td>MMP-1</td>
<td>4.6</td>
<td>5.4</td>
</tr>
<tr>
<td>(4.4–6.4)</td>
<td>(2.3–6.4)</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>991</td>
<td>921</td>
</tr>
<tr>
<td>(787–1088)</td>
<td>(719–1088)</td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>10.0</td>
<td>10.2</td>
</tr>
<tr>
<td>(7.3–14.4)</td>
<td>(7.3–14.1)</td>
<td></td>
</tr>
<tr>
<td>MMP-1/TIMP-1</td>
<td>complex 2.9</td>
<td>2.2</td>
</tr>
<tr>
<td>(1.7–4.1)</td>
<td>(0.4–4.7)</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>249</td>
<td>254</td>
</tr>
<tr>
<td>(182–362)</td>
<td>(182–351)</td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>499</td>
<td>532</td>
</tr>
<tr>
<td>(469–583)</td>
<td>(475–584)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Logistic regression analysis revealed that the only significant predictor of embolisation was a plasma MMP-9 level of >30.2 ng/ml (the median value for the entire patient cohort), equating to a relative risk of 2.54.

<table>
<thead>
<tr>
<th>Risk factor for embolisation</th>
<th>( p )-value</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma [MMP-9] &gt;30.2 ng/ml</td>
<td>0.05</td>
<td>2.54</td>
</tr>
<tr>
<td>Age &gt;68 years</td>
<td>0.70</td>
<td>1.02</td>
</tr>
<tr>
<td>Sex</td>
<td>0.64</td>
<td>0.90</td>
</tr>
<tr>
<td>Symptoms</td>
<td>0.09</td>
<td>2.40</td>
</tr>
<tr>
<td>Current/past smoker</td>
<td>0.92</td>
<td>0.98</td>
</tr>
<tr>
<td>IHD</td>
<td>0.10</td>
<td>2.0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.30</td>
<td>0.96</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.95</td>
<td>1.24</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>0.08</td>
<td>0.67</td>
</tr>
</tbody>
</table>
symptoms and a history of ischaemic heart disease increased the risk of embolisation, they failed to reach significance.

All patients made uneventful postoperative recoveries with no perioperative strokes or deaths.

**Discussion**

The present study showed increased plasma levels of MMP-9 in those patients with significant carotid stenoses undergoing carotid endarterectomy in whom spontaneous particulate embolisation was detected. There were no differences in the circulating levels of other MMPs or their naturally occurring inhibitors, TIMPs-1 and -2. This suggests a shift in the balance of circulating proteolytic enzymes towards proteolysis, a process that is usually tightly controlled.

This may represent one of two phenomena. Firstly, it may reflect a systemic manifestation of the localised increase in MMP-9 levels and activity previously demonstrated within unstable plaques, occurring in tandem with intense localised inflammation and tissue degradation. MMPs are now recognised to play a key role in many aspects of the atherosclerotic process, but particularly the onset of instability and plaque rupture. The intraplaque level, activity and expression of MMP-9 has been shown to be significantly higher in the most unstable plaques based on patient symptomatology, spontaneous embolisation and histological features on instability. There may be an increased release of the enzyme into the circulation from the plaque itself, or by circulating macrophages involved in the inflammatory response promoting plaque degradation. In acute coronary syndromes, for example, monocytes in the systemic circulation undergo activation and may represent a source of enzyme.

The increase may on the other hand represent a marker of end organ ischaemia in the form of thrombo-embolic cerebral damage. CT scans were not performed in this study to look specifically for evidence of infarction because previous studies of particulate embolisation have found that CT is unhelpful in this regard. A more sensitive measure of end organ damage may be psychometric testing and previous studies have shown a clear link between particulate embolisation and a deterioration in psychological scoring.

Experimental models of focal cerebral ischaemia have shown an association between the degree of cerebral injury and cerebral MMP levels. Middle cerebral artery occlusion in rats has been shown to cause an increased MMP-2 and MMP-9 activity, whilst further studies have shown that MMP-9 may contribute to post-infarction oedema and haemorrhage. More recently, MMP-2 and MMP-9 levels have been demonstrated to increase very early within ischaemic basal ganglia of non-human primates in parallel with evidence of neurone injury. In humans the level of cerebral MMP-9 has been shown to be high in association with an inflammatory infiltration predominantly of leukocytes in patients who died shortly after a stroke.

Increased cerebral levels of MMP-9 may therefore contribute to early disruption of the microvascular basal lamina and accentuate neuronal injury. The cellular source of cerebral MMPs remains unclear but focal cerebral ischaemia may influence plasma MMP levels and vice versa.

Ji Hoe Heo and colleagues demonstrated a transient rise in circulating MMP-9 levels in a rat model of middle cerebral artery occlusion. However, there are no previous reports linking plasma MMP levels with markers of plaque instability. Circulating levels of both MMP-2 and MMP-9 have been shown to increase in patients with acute coronary syndromes. Kai et al. measured plasma MMP levels in 33 patients with acute coronary syndromes, 17 patients with stable angina and 17 normal controls. Early increases in circulating levels of both MMP-2 and MMP-9 were demonstrated in patients with acute coronary syndromes, higher than both normal controls and those with stable angina. These results support those of the current paper, though we recognise the possibility of a chance finding and the need for larger scale studies.

Further investigations are required to address the cellular source of the increased circulating MMP-9 levels observed in embolising patients. However, it may represent a useful marker of plaque instability or the general level of atherosclerosis-related inflammation and perhas identify a cohort of patients who warrant urgent intervention.

**Acknowledgements**

Funding from the Stroke Association and The Royal College of Surgeons of England.

**References**


Eur J Vasc Endovasc Surg Vol 21, January 2001
Plasma MMP-9


14 Van der Wal AC, Becker AE, Van Der Loos CM, Das P. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterised by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994; 90: 36–44.


